IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Sinskey *et al.*	Art Unit: To be Assigned
Appl. No. To be assigned (Divisional of U.S. Appl. No. 09/677,575; Filed: October 3, 2000)	Examiner: To be Assigned
Filed: Herewith	Atty. Docket: 1533.0790002/MAC/AGU
For: **Pyruvate Carboxylase from *Corynebacterium glutamicum***	

Preliminary Amendment and Submission of Sequence Listing

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination on the merits, kindly amend the captioned application as follows. This Preliminary Amendment is provided in the following format:

(A) A clean version of each replacement paragraph/section/claim along with clear instructions for entry;

(B) Starting on a separate page, appropriate remarks and arguments. *See* 37 C.F.R. § 1.121 and MPEP 714; and

(C) Starting on a separate page, a marked-up version entitled: "Version with markings to show changes made."

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including

fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Amendments

In the Specification:

Please insert the sequence listing at the end of the application.

Please substitute the paragraph beginning on page 2, line 21, with the following paragraph:

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a pyruvate carboxylase polypeptide having the amino acid sequence in Figures 1A-C (SEQ ID NO:2) or the amino acid sequence encoded by the clone deposited in a bacterial host as ATCC Deposit Number PTA-982. The nucleotide sequence determined by sequencing the deposited pyruvate carboxylase clone, which is shown in Figures 1A-C (SEQ ID NO:1), contains an open reading frame encoding a polypeptide of 1140 amino acid residues which has a deduced molecular weight of about 123.6 kDa. The 1140 amino acid sequence of the predicted pyruvate carboxylase protein is shown in Figures 1A-C and in SEQ ID NO:2.

Please substitute the paragraph beginning on page 2, line 31, with the following paragraph:

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the clone contained in ATCC Deposit No. PTA-982; and (c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b) above.

Please substitute the paragraph beginning on page 3, line 21, with the following paragraph:

The invention further provides an isolated pyruvate carboxylase polypeptide having amino acid sequence selected from the group consisting of: (a) the amino acid sequence of the pyruvate carboxylase polypeptide having the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2); and (b) the amino acid sequence of the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the clone contained in ATCC Deposit No. PTA-982. The polypeptides of the present invention also include polypeptides having an amino acid sequence with at least 90% similarity, more preferably at least 95% similarity to those described in (a) or (b) above, as well as polypeptides having an amino acid sequence at least 70% identical, more preferably at least 90% identical, and still more preferably 95%, 97%, 98% or 99% identical to those above.

Please substitute the paragraph beginning on page 4, line 3, with the following paragraph:

Figures 1A-C show the nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences of the complete pyruvate carboxylase protein determined by sequencing of the DNA clone contained in ATCC Deposit No. PTA-982. The protein has sequence of about 1140 amino acid residues and a deduced molecular weight of about 123.6 kDa.

Please substitute the paragraph beginning on page 4, line 11, with the following paragraph:

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding the pyruvate carboxylase protein having the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2) which was determined by sequencing a cloned cosmid. The pyruvate carboxylase protein of the present invention shares sequence homology with *M. tuberculosis* and human pyruvate carboxylase proteins. The nucleotide sequence shown in Figures 1A-C (SEQ ID NO:1) was obtained by sequencing cosmid III F10 encoding a pyruvate carboxylase polypeptide. A clone containing the pyruvate carboxylate gene was deposited on November 22, 1999 at the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, and given accession number PTA-982.

Please substitute the paragraph beginning on page 5, line 22, with the following paragraph:

Using the information provided herein, such as the nucleotide sequence in Figures 1A-C, a nucleic acid molecule of the present invention encoding a pyruvate carboxylase polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning DNAs using mRNA as starting material. The pyruvate carboxylase protein shown in Figures 1A-C (SEQ ID NO:2) is about 63% identical to *M. tuberculosis* and 44% identical to human. As one of ordinary skill would appreciate, due to the possibilities of sequencing errors discussed above, as well as the variability of cleavage sites for leaders in different known proteins, the actual pyruvate carboxylase polypeptide encoded by the deposited clone comprises about 1140 amino acids, but may be anywhere in the range of 1133-1147 amino acids.

Please substitute the paragraph beginning on page 6, line 16, with the following paragraph:

Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) with an initiation codon at positions 199-201 of the nucleotide sequence shown in Figures 1A-C (SEQ ID NO:1); DNA molecules comprising the coding sequence for the pyruvate carboxylase protein shown in Figures 1A-C and SEQ ID NO:2; and DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the pyruvate carboxylase protein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above.

Please substitute the paragraph beginning on page 6, line 25, with the following paragraph:

In another aspect, the invention provides isolated nucleic acid molecules encoding the pyruvate carboxylase polypeptide having an amino acid sequence encoded by the clone deposited as ATCC Deposit No. PTA-982. Preferably, this nucleic acid molecule will encode the polypeptide encoded by the above-described deposited clone. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in Figures 1A-C (SEQ ID NO:1) or the

nucleotide sequence of the pyruvate carboxylase DNA contained in the above-described deposited clone, or nucleic acid molecule having a sequence complementary to one of the above sequences.

Please substitute the paragraph beginning on page 7, line 2, with the following paragraph:

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the clone contained in ATCC Deposit PTA-982. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers.

Please substitute the paragraph beginning on page 7, line 16, with the following paragraph:

Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide (e.g., the deposited clone), for instance, a portion 50-750 nt in length, or even to the entire length of the reference polynucleotide, also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of the nucleotide sequence of the deposited DNA or the nucleotide sequence as shown in Figures 1A-C (SEQ ID NO:1). By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide, (e.g., the deposited DNA or the nucleotide sequence as shown in Figures 1A-C (SEQ ID NO:1)). As indicated, such portions are useful diagnostically either as a probe according to conventional DNA hybridization techniques or as primers for amplification of a target sequence by the polymerase chain reaction (PCR), as described, for instance, in *Molecular Cloning, A Laboratory Manual*, 2nd. edition, edited by

Sambrook, J., Fritsch, E. F. and Maniatis, T., (1989), Cold Spring Harbor Laboratory Press, the entire disclosure of which is hereby incorporated herein by reference.

Please substitute the paragraph beginning on page 7, line 32, with the following paragraph:

Since a pyruvate carboxylase clone has been deposited and its determined nucleotide sequence is provided in Figures 1A-C (SEQ ID NO:1), generating polynucleotides which hybridize to a portion of the pyruvate carboxylase DNA molecule would be routine to the skilled artisan. For example, restriction endonuclease cleavage or shearing by sonication of the pyruvate carboxylase clone could easily be used to generate DNA portions of various sizes which are polynucleotides that hybridize to a portion of the pyruvate carboxylase DNA molecule. Alternatively, the hybridizing polynucleotides of the present invention could be generated synthetically according to known techniques.

Please substitute the paragraph beginning on page 9, line 6, with the following paragraph:

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the pyruvate carboxylase protein or portions thereof. Also especially preferred in this regard are conservative substitutions. Most highly preferred are nucleic acid molecules encoding the pyruvate carboxylase protein having the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2).

Please substitute the paragraph beginning on page 9, line 19, with the following paragraph:

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded

by the clone contained in ATCC Deposit No. PTA-982; or (c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b).

Please substitute the paragraph beginning on page 10, line 11, with the following paragraph:

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figures 1A-C or to the nucleotides sequence of the deposited clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman (*Advances in Applied Mathematics 2*: 482-489 (1981)) to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

Please substitute the paragraph beginning on page 10, line 25, with the following paragraph:

The present application is directed to nucleic acid molecules at least 90%, 95%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figures 1A-C (SEQ ID NO:1) or to the nucleic acid sequence of the deposited DNA, irrespective of whether they encode a polypeptide having pyruvate carboxylase activity. This is because, even where a particular nucleic acid molecule does not encode a polypeptide having pyruvate carboxylase activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer.

Please substitute the paragraph beginning on page 11, line 1, with the following paragraph:

Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figures 1A-C (SEQ ID NO:1) or to the

nucleic acid sequence of the deposited DNA which do, in fact, encode a polypeptide having pyruvate carboxylase protein activity. By "a polypeptide having pyruvate carboxylase activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the pyruvate carboxylase protein of the invention as measured in a particular biological assay.

Please substitute the paragraph beginning on page 11, line 9, with the following paragraph:

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited DNA or the nucleic acid sequence shown in Figures 1A-C (SEQ ID NO:1) will encode a polypeptide "having pyruvate carboxylase protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having pyruvate carboxylase protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

Please substitute the paragraph beginning on page 15, line 15, with the following paragraph:

The invention further provides an isolated pyruvate carboxylase polypeptide having the amino acid sequence encoded by the deposited DNA, or the amino acid sequence in Figures 1A-C (SEQ ID NO:2), or a peptide or polypeptide comprising a portion of the above polypeptides. The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least to amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than ten amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus.

Please substitute the paragraph beginning on page 17, line 22, with the following paragraph:

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2) or to the amino acid sequence encoded by deposited clone can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

Please substitute the paragraph beginning on page 24, line 29, with the following paragraph:

PCR was performed using the Boehringer Mannheim PCR core kit following the manufacturer's instructions. When PCR was performed on *Corynebacterium* chromosomal DNA, about 1 μg DNA was used in each reaction. The forward primer used was 5′GTCTTCATCGAGATGAATCCGCG3′ (SEQ ID NO:3) and the reverse primer used was 5′CGCAGCGCCACATCGTAAGTCGC3′ (SEQ ID NO:4) for the PCR reaction.

Please substitute table 1 beginning on page 27, line 16, with the following table 1:

Organism	Conserved region A	Conserved region B
Caenorhabditis elegans	YFIEVNAR (SEQ ID NO:5)	ATFDVSM (SEQ ID NO:6)
Aedes aegypti	YFIEVNAR (SEQ ID NO:7)	ATFDVAL (SEQ ID NO:8)
Mycobacterium tuberculosis	VFIEMNPR (SEQ ID NO:9)	ATYDVAL (SEQ ID NO:10)
Bacillus stearothermophilus	YFIEVNPR (SEQ ID NO:11)	ATFDVAY (SEQ ID NO:12)
Pichia pastoris	YFIEINPR (SEQ ID NO:13)	ATFDVSM (SEQ ID NO:14)
Mus musculus	YFIEVNSR (SEQ ID NO:15)	ATFDVAM (SEQ ID NO:16)
Rattus norvegicus	YFIEVNSR (SEQ ID NO:17)	ATFDVAM (SEQ ID NO:18)
Saccharomyces cerevisiae 1	YFIEINPR (SEQ ID NO:19)	ATFDVAM (SEQ ID NO:20)
Saccharomyces cerevisiae 2	YFIEINPR (SEQ ID NO:21)	ATFDVAM (SEQ ID NO:22)
Rhizabium etli	YFIEVNPR (SEQ ID NO:23)	ATFDVSM (SEQ ID NO:24)
Homo sapiens	YFIEVNSR (SEQ ID NO:25)	ATFDVAM (SEQ ID NO:26)
Schizosaccharomyces pombe	YFIEINPR (SEQ ID NO:27)	ATFDVSM (SEQ ID NO:28)

Please substitute the paragraph beginning on page 28, line 25, with the following paragraph:

The amino-terminal segment of the *C. glutamicum* pyruvate carboxylase contains the hexapeptide GGGGRG (SEQ ID NO:37), which matches the GGGG(R/K)G (SEQ ID NO:38) sequence that is found in all biotin-binding proteins and is believed to be an ATP-binding site (Fry, D.C., *et al., Proc. Natl. Acad. Sci. USA 83*:907-911 (1986); Post, L.E., *et al., J. Biol. Chem. 265*:7742-7747 (1990)). A second region that is proposed to be involved in ATP binding and is present in biotin-dependent carboxylases and carbamyphosphate synthetase (Lim, F., *et al., J. Biol. Chem. 263*:11493-11497 (1988)) is conserved in the *C. glutamicum* sequence. The predicted *C. glutamicum* pyruvate carboxylase protein also contains a putative pyruvate-binding motif, FLFEDPWDR (SEQ ID NO:29), which is conserved in the transcarboxylase domains of *Mycobacterium, Rhizobium* and human pyruvate carboxylases (Dunn, M.F., *et al., J. Bacteriol. 178*:5960-5970 (1996)). Tryptophan fluorescence studies with transcarboxylase have shown that the Trp residue present in this motif is involved in pyruvate binding (Kumer, G.K., *et al., Biochemistry 27*:5978-5983 (1988)). The carboxy-terminal segment of the enzyme contains a putative biotin-binding site, AMKM (SEQ ID NO:39), which is identical to those found in other pyruvate carboxylases as well as the biotin-carboxyl-carrier protein (BCCP) domains of other biotin-dependent enzymes.

Please substitute table 2 beginning on page 29, line 9, with the following table 2:

Primer name	*Primer sequence (5'-3')*
Begrev1	*TTCACCAGGTCCACCTCG* (SEQ ID NO:30)
Endfor1	*CGTCGCAAAGCTGACTCC* (SEQ ID NO:31)
Begrev2	*GATGCTTCTGTTGCTAATTTGC* (SEQ ID NO:32)
Endfor2	*GGCCATTAAGGATATGGCTG* (SEQ ID NO:33)
Begrev3	*GCGGTGGAATGATCCCCGA* (SEQ ID NO:34)
Endfor3	*ACCGCACTGGGCCTTGCG* (SEQ ID NO:35)
Endfor4	*TCGCCGCTTCGGCAACAC* (SEQ ID NO:36)

In the Claims:

Please cancel claims 1-13 without prejudice to or disclaimer of the subject matter encompassed thereby.

Please amend the claims as follows:

14. (Once Amended) An isolated pyruvate carboxylase polypeptide having an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the amino acid sequence of the pyruvate carboxylase polypeptide having the complete amino acid sequence in SEQ ID NO:2; and

(b) the amino acid sequence of the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the clone contained in ATCC Deposit No. PTA-982.

15. (Once Amended) A method of making amino acids comprising expressing an isolated nucleic acid molecule encoding pyruvate carboxylase comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the amino acid sequence in SEQ ID NO:2;

(b) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the clone contained in ATCC Deposit No. PTA-982; and

(c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b), and recovering said amino acids.

Remarks

In the documents accompanying this Preliminary Amendment, claims 1-13 have been canceled. Upon entry of the foregoing amendment, claims 14-17 are pending in the application, with claims 14 and 15 being the independent claims. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Applicants have amended the specification to refer to the ATCC Deposit No. of a deposited plasmid clone containing the pyruvate carboxylase gene and to direct the entry of the formal drawings where appropriate. Applicants have amended the specification to delete reference to a deposited "cosmid" clone. The specification has also been amended to direct the entry of this sequence listing after the claims of the above identified application and to provide the SEQ ID NO's next to the specific sequence. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Claim 14 has been amended to correct a typographical error and is believed to introduce no new matter. Claim 15 has been amended to delete reference to a canceled claim, and to insert the contents of the canceled claim.

In accordance with 37 C.F.R. § 1.821(g), this submission includes no new matter.

In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Sequence Listing and the computer readable copy of the Sequence Listing submitted herewith in the above application are the same.

Conclusion

Applicants believe the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Preliminary Amendment is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Michele A. Cimbala
Attorney for Applicants
Registration No. 33,851

Date: January 15, 2002

1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934
(202) 371-2600

P:\USERS\ANGIE\1533\0790002\prelimamdt.wpd

Version with markings to show changes made

In the Specification:

Please substitute the paragraph beginning on page 2, line 21, with the following paragraph:

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a pyruvate carboxylase polypeptide having the amino acid sequence in Figures 1A-C (SEQ ID NO:2) or the amino acid sequence encoded by the [cosmid] clone deposited in a bacterial host as ATCC Deposit Number [____] PTA-982. The nucleotide sequence determined by sequencing the deposited pyruvate carboxylase [cosmid] clone, which is shown in Figures 1A-C (SEQ ID NO:1), contains an open reading frame encoding a polypeptide of 1140 amino acid residues which has a deduced molecular weight of about 123.6 kDa. The 1140 amino acid sequence of the predicted pyruvate carboxylase protein is shown in Figures 1A-C and in SEQ ID NO:2.

Please substitute the paragraph beginning on page 2, line 31, with the following paragraph:

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the [cosmid] clone contained in ATCC Deposit No. [____] PTA-982; and (c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b) above.

Please substitute the paragraph beginning on page 3, line 21, with the following paragraph:

The invention further provides an isolated pyruvate carboxylase polypeptide having amino acid sequence selected from the group consisting of: (a) the amino acid sequence of the pyruvate carboxylase polypeptide having the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2); and (b) the amino acid sequence of the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the [cosmid] clone contained in ATCC Deposit No. [____] PTA-982. The polypeptides of the present invention also include polypeptides having an amino acid

sequence with at least 90% similarity, more preferably at least 95% similarity to those described in (a) or (b) above, as well as polypeptides having an amino acid sequence at least 70% identical, more preferably at least 90% identical, and still more preferably 95%, 97%, 98% or 99% identical to those above.

Please substitute the paragraph beginning on page 4, line 3, with the following paragraph:

Figures 1A-C show[s] the nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences of the complete pyruvate carboxylase protein determined by sequencing of the DNA clone contained in ATCC Deposit No. [_____] PTA-982. The protein has sequence of about 1140 amino acid residues and a deduced molecular weight of about 123.6 kDa.

Please substitute the paragraph beginning on page 4, line 11, with the following paragraph:

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding the pyruvate carboxylase protein having the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2) which was determined by sequencing a cloned cosmid. The pyruvate carboxylase protein of the present invention shares sequence homology with *M. tuberculosis* and human pyruvate carboxylase proteins. The nucleotide sequence shown in Figures 1A-C (SEQ ID NO:1) was obtained by sequencing cosmid III F10 encoding a pyruvate carboxylase polypeptide. [, which] A clone containing the pyruvate carboxylate gene was deposited on [___] November 22, 1999 at the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, and given accession number [_____] PTA-982.

Please substitute the paragraph beginning on page 5, line 22, with the following paragraph:

Using the information provided herein, such as the nucleotide sequence in Figures 1A-C, a nucleic acid molecule of the present invention encoding a pyruvate carboxylase polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning DNAs using mRNA as starting material. The pyruvate carboxylase protein shown in Figures 1A-C (SEQ ID NO:2) is about 63% identical to *M. tuberculosis* and 44% identical to human. As one of ordinary skill would appreciate, due to the possibilities of sequencing errors discussed above, as well as the

variability of cleavage sites for leaders in different known proteins, the actual pyruvate carboxylase polypeptide encoded by the deposited [cosmid] clone comprises about 1140 amino acids, but may be anywhere in the range of 1133-1147 amino acids.

Please substitute the paragraph beginning on page 6, line 16, with the following paragraph:

Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) with an initiation codon at positions 199-201 of the nucleotide sequence shown in Figures 1A-C (SEQ ID NO:1); DNA molecules comprising the coding sequence for the pyruvate carboxylase protein shown in Figures 1A-C and SEQ ID NO:2; and DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the pyruvate carboxylase protein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above.

Please substitute the paragraph beginning on page 6, line 25, with the following paragraph:

In another aspect, the invention provides isolated nucleic acid molecules encoding the pyruvate carboxylase polypeptide having an amino acid sequence encoded by the [cosmid] clone deposited as ATCC Deposit No. [_____] PTA-982. Preferably, this nucleic acid molecule will encode the polypeptide encoded by the above-described deposited clone. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in Figures 1A-C (SEQ ID NO:1) or the nucleotide sequence of the pyruvate carboxylase DNA contained in the above-described deposited clone, or nucleic acid molecule having a sequence complementary to one of the above sequences.

Please substitute the paragraph beginning on page 7, line 2, with the following paragraph:

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the [cosmid] clone contained in ATCC Deposit [_____] PTA-982. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x

SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers.

Please substitute the paragraph beginning on page 7, line 16, with the following paragraph:

Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide (e.g., the deposited [cosmid] clone), for instance, a portion 50-750 nt in length, or even to the entire length of the reference polynucleotide, also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of the nucleotide sequence of the deposited DNA or the nucleotide sequence as shown in Figures 1A-C (SEQ ID NO:1). By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide, (e.g., the deposited DNA or the nucleotide sequence as shown in Figures 1A-C (SEQ ID NO:1)). As indicated, such portions are useful diagnostically either as a probe according to conventional DNA hybridization techniques or as primers for amplification of a target sequence by the polymerase chain reaction (PCR), as described, for instance, in *Molecular Cloning, A Laboratory Manual*, 2nd. edition, edited by Sambrook, J., Fritsch, E. F. and Maniatis, T., (1989), Cold Spring Harbor Laboratory Press, the entire disclosure of which is hereby incorporated herein by reference.

Please substitute the paragraph beginning on page 7, line 32, with the following paragraph:

Since a pyruvate carboxylase [cosmid] clone has been deposited and its determined nucleotide sequence is provided in Figures 1A-C (SEQ ID NO:1), generating polynucleotides which hybridize to a portion of the pyruvate carboxylase DNA molecule would be routine to the skilled artisan. For example, restriction endonuclease cleavage or shearing by sonication of the pyruvate carboxylase [cosmid] clone could easily be used to generate DNA portions of various sizes which

are polynucleotides that hybridize to a portion of the pyruvate carboxylase DNA molecule. Alternatively, the hybridizing polynucleotides of the present invention could be generated synthetically according to known techniques.

Please substitute the paragraph beginning on page 9, line 6, with the following paragraph:

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the pyruvate carboxylase protein or portions thereof. Also especially preferred in this regard are conservative substitutions. Most highly preferred are nucleic acid molecules encoding the pyruvate carboxylase protein having the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2).

Please substitute the paragraph beginning on page 9, line 19, with the following paragraph:

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the [cosmid] clone contained in ATCC Deposit No. [_____] PTA-982; or (c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b).

Please substitute the paragraph beginning on page 10, line 11, with the following paragraph:

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figures 1A-C or to the nucleotides sequence of the deposited [cosmid] clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package,

Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman (*Advances in Applied Mathematics 2*: 482-489 (1981)) to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

Please substitute the paragraph beginning on page 10, line 25, with the following paragraph:

The present application is directed to nucleic acid molecules at least 90%, 95%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figures 1A-C (SEQ ID NO:1) or to the nucleic acid sequence of the deposited DNA, irrespective of whether they encode a polypeptide having pyruvate carboxylase activity. This is because, even where a particular nucleic acid molecule does not encode a polypeptide having pyruvate carboxylase activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer.

Please substitute the paragraph beginning on page 11, line 1, with the following paragraph:

Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figures 1A-C (SEQ ID NO:1) or to the nucleic acid sequence of the deposited DNA which do, in fact, encode a polypeptide having pyruvate carboxylase protein activity. By "a polypeptide having pyruvate carboxylase activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the pyruvate carboxylase protein of the invention as measured in a particular biological assay.

Please substitute the paragraph beginning on page 11, line 9, with the following paragraph:

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least

90%, 95%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited DNA or the nucleic acid sequence shown in Figures 1A-C (SEQ ID NO:1) will encode a polypeptide "having pyruvate carboxylase protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having pyruvate carboxylase protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

Please substitute the paragraph beginning on page 15, line 15, with the following paragraph:

The invention further provides an isolated pyruvate carboxylase polypeptide having the amino acid sequence encoded by the deposited DNA, or the amino acid sequence in Figures 1A-C (SEQ ID NO:2), or a peptide or polypeptide comprising a portion of the above polypeptides. The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least to amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than ten amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus.

Please substitute the paragraph beginning on page 17, line 22, with the following paragraph:

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figures 1A-C (SEQ ID NO:2) or to the amino acid sequence encoded by deposited [cosmid] clone can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of

identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

Please substitute the paragraph beginning on page 24, line 29, with the following paragraph:

PCR was performed using the Boehringer Mannheim PCR core kit following the manufacturer's instructions. When PCR was performed on *Corynebacterium* chromosomal DNA, about 1 μg DNA was used in each reaction. The forward primer used was 5'GTCTTCATCGAGATGAATCCGCG3' (SEQ ID NO:3) and the reverse primer used was 5'CGCAGCGCCACATCGTAAGTCGC3' (SEQ ID NO:4) for the PCR reaction.

Please substitute table 1 beginning on page 27, line 16, with the following table 1:

Organism	**Conserved region A**	**Conserved region B**
Caenorhabditis elegans	YFIEVNAR (SEQ ID NO:5)	ATFDVSM (SEQ ID NO:6)
Aedes aegypti	YFIEVNAR (SEQ ID NO:7)	ATFDVAL (SEQ ID NO:8)
Mycobacterium tuberculosis	VFIEMNPR (SEQ ID NO:9)	ATYDVAL (SEQ ID NO:10)
Bacillus stearothermophilus	YFIEVNPR (SEQ ID NO:11)	ATFDVAY (SEQ ID NO:12)
Pichia pastoris	YFIEINPR (SEQ ID NO:13)	ATFDVSM (SEQ ID NO:14)
Mus musculus	YFIEVNSR (SEQ ID NO:15)	ATFDVAM (SEQ ID NO:16)
Rattus norvegicus	YFIEVNSR (SEQ ID NO:17)	ATFDVAM (SEQ ID NO:18)
Saccharomyces cerevisiae 1	YFIEINPR (SEQ ID NO:19)	ATFDVAM (SEQ ID NO:20)
Saccharomyces cerevisiae 2	YFIEINPR (SEQ ID NO:21)	ATFDVAM (SEQ ID NO:22)
Rhizabium etli	YFIEVNPR (SEQ ID NO:23)	ATFDVSM (SEQ ID NO:24)
Homo sapiens	YFIEVNSR (SEQ ID NO:25)	ATFDVAM (SEQ ID NO:26)
Schizosaccharomyces pombe	YFIEINPR (SEQ ID NO:27)	ATFDVSM (SEQ ID NO:28)

Please substitute the paragraph beginning on page 28, line 25, with the following paragraph:

The amino-terminal segment of the *C. glutamicum* pyruvate carboxylase contains the hexapeptide GGGGRG (SEQ ID NO:37), which matches the GGGG(R/K)G (SEQ ID NO:38) sequence that is found in all biotin-binding proteins and is believed to be an ATP-binding site (Fry, D.C., *et al., Proc. Natl. Acad. Sci. USA 83*:907-911 (1986); Post, L.E., *et al., J. Biol. Chem. 265*:7742-7747 (1990)). A second region that is proposed to be involved in ATP binding and is

present in biotin-dependent carboxylases and carbamyphosphate synthetase (Lim, F., *et al., J. Biol. Chem. 263*:11493-11497 (1988)) is conserved in the *C. glutamicum* sequence. The predicted *C. glutamicum* pyruvate carboxylase protein also contains a putative pyruvate-binding motif, FLFEDPWDR (SEQ ID NO:29), which is conserved in the transcarboxylase domains of *Mycobacterium, Rhizobium* and human pyruvate carboxylases (Dunn, M.F., *et al., J. Bacteriol. 178*:5960-5970 (1996)). Tryptophan fluorescence studies with transcarboxylase have shown that the Trp residue present in this motif is involved in pyruvate binding (Kumer, G.K., *et al., Biochemistry 27*:5978-5983 (1988)). The carboxy-terminal segment of the enzyme contains a putative biotin-binding site, AMKM (SEQ ID NO:39), which is identical to those found in other pyruvate carboxylases as well as the biotin-carboxyl-carrier protein (BCCP) domains of other biotin-dependent enzymes.

Please substitute table 2 beginning on page 29, line 9, with the following table 2:

Primer name	*Primer sequence (5'-3')*
Begrev1	*TTCACCAGGTCCACCTCG* (SEQ ID NO:30)
Endfor1	*CGTCGCAAAGCTGACTCC* (SEQ ID NO:31)
Begrev2	*GATGCTTCTGTTGCTAATTTGC* (SEQ ID NO:32)
Endfor2	*GGCCATTAAGGATATGGCTG* (SEQ ID NO:33)
Begrev3	*GCGGTGGAATGATCCCCGA* (SEQ ID NO:34)
Endfor3	*ACCGCACTGGGCCTTGCG* (SEQ ID NO:35)
Endfor4	*TCGCCGCTTCGGCAACAC* (SEQ ID NO:36)

In the Claims:

(a) Claims 1-13 have been cancelled.

(b) Claims 14 and 15 are amended as follows:

14. (Once Amended) An isolated pyruvate carboxylase polypeptide having an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the amino acid sequence of the pyruvate carboxylase polypeptide having the complete amino acid sequence in SEQ ID NO:2; and

(b) the amino acid sequence of the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the [cosmid] clone contained in ATCC Deposit No.[_____; and] PTA-982.

15. (Once Amended) A method of making amino acids comprising expressing [the nucleotide sequence of claim 1] an isolated nucleic acid molecule encoding pyruvate carboxylase comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the amino acid sequence in SEQ ID NO:2;

(b) a nucleotide sequence encoding the pyruvate carboxylase polypeptide having the complete amino acid sequence encoded by the clone contained in ATCC Deposit No. PTA-982; and

(c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b), and recovering said amino acids.